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MOLECULAR CLONING AND FUNCTIONAL EXPRESSION OF α-BUNGAROTOXIN (V31) FROM CHINESE CONTINENTAL BANDED KRAIT①

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Abstract: The cDNA encoding a variant of α-bungarotoxin was cloned from the venom glands of *Bungarus multicinctus* by RT-PCR. The deduced protein precursor contained a 21 amino acid signal peptide and a following 74 amino acid mature protein. The signal peptide is very similar to those of short chain neurotoxins, κ-neurotoxins and cardiotoxins. The amino acid sequence of the mature protein is identical to α-bungarotoxin (V31), a minor variant of α-bungarotoxin identified by protein sequencing technique. Furthermore, the cDNA encoding the deletion precursor of α-bungarotoxin was also cloned. By use of pMAL-p2, the variant was overexpressed in *E. coli* as a soluble fusion protein and purified by sepharose 6B-amylose affinity chromatography, which was confirmed by western blotting with the antisera against α-bungarotoxin. The recombinant variant was achieved after digestion by factor X_a. It displayed about 1/6 *in vivo* toxicity of natural α-bungarotoxin. The successful cloning and functional expression of α-bungarotoxin provided a basis for the future study of structure-function of long neurotoxins.

Key words: α-bungarotoxin (V31); cDNA cloning; Fusion expression; *In vivo* toxicity

One of the most intensively studied long neurotoxins is α-bungarotoxin, which is identified from the venom of *Bungarus multicinctus*. It has played a critically important role in the location, purification and functional characterization of nicotine acetylcholine receptors (nAChRs) (Stroud *et al.*, 1990; Changeux *et al.*, 1992). X-ray crystal structure and two-dimensional NMR of α-bungarotoxin have been reported (Love *et al.*, 1986; Basus *et al.*, 1988; Kosen *et al.*, 1988). α-Bungarotoxin comprises of 74 amino acid residues and five disulfide bonds with molecular weight of 8 kD approx. A minor variant of α-bungarotoxin is also identified by protein sequencing technique, in which the residue Ala-31 of α-bungarotoxin is substituted by Val-31 (Kosen *et al.*, 1988). In the past, some residues of several α-neurotoxins implicated in the binding with nAChR were tentatively identified by sequence comparisons and conventional chemical modifications (Endo, 1991). Recently, a synthetic gene has been cloned to produce active recombinant α-bungarotoxin (Rosenthal *et al.*, 1994). Furthermore, the cDNA encoding α-bungarotoxin has also been cloned by two steps of RT-PCR based on its amino acid sequence (Kuo *et al.*, 1995). However, in this investigation the cDNA encoding the precursor of α-bungarotoxin (V31), the variant of α-bungarotoxin, was cloned by one step of RT-PCR from the venom gland of the Chinese continental banded krait. The variant was overexpressed in *E. coli* and purified as a fusion protein. After digestion by factor X_a, the achieved recombinant variant showed the biological activity.

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①The sequence data of the cDNAs for the precursor and the deletion precursor of the α-bungarotoxin (V31) have been deposited in the EMBL data library under the accession number AJ007766 and AJ131356
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1 Materials and Methods

1.1 Preparation of total RNA from venom gland

The head of *Bungarus multicinctus multicinctus* was cut off and the venom glands were taken out immediately, stored at −70℃. One venom gland was homogenized to extract the total RNA by a guanidinium isothiocyanate/phenol chloroform isolation kit (Promega, USA).

1.2 Synthesis of single strand cDNA

2 μg above prepared RNA template and 30 ng synthesized oligo $(dT)_{25}$ antisense primer were mixed with diethyl pyrocarbonate (DEPC) treated water to a final volume of 15 μL. The mixture was then treated at 70℃ for 5 min to disrupt the secondary structure within the RNA template and cooled immediately on ice to prevent the secondary structure from reforming. After that, 5 × RT buffer, 0.5 mmol/L dNTP, 20 U RNasin inhibitor and 200 U M-MLV reverse transcriptase were added to a total volume of 25 μL and then incubated at 42℃ for 1 h.

1.3 PCR amplification and cloning

Two degenerate primers were designed by comparison of reported cDNA sequences of neurotoxins from Elapidae and Hydrophiidae snake venoms, whose 5′ and 3′ untranslated regions were highly conserved. The plus primer was 5′ − AGATGAAAACTCTG(C/T) TGCTG (A/T) CCTTGG − 3′ and the minus primer was 5′ − GGATGGTCCATGAT (T/G) GA (T/G) GAGAGCAA − 3′. The primers were synthesized by Oligo 1 000 DNA synthesizer (Beckman Inc., USA). PCR was carried out with a 100 μL reaction buffer containing 50 mmol/L KCl, 10 mmol/L Tris·Cl (pH 9.0), 0.1% Triton X − 100, 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTP mixtures, 1 μmol/L plus and minus primer each, 2 μL reverse transcription cDNA and 2.5 U DNA polymerase (AGCT Inc., Canada). It was performed on a thermocycler at the following steps: 94℃, denatured for 5 min; then 94℃/55℃/72℃ 1 min each for 35 cycles; 72℃ extension for 10 min at last. The PCR products were purified by Wizard PCR preps kit (Promega, USA) and cloned into the pGEMT-vector (Promega, USA).

1.4 DNA sequencing and sequence analysis

DNA sequencing was performed on an ABI 373 automatic DNA sequencer (Applied Biosystems Inc., USA) with T_7 primer. The deduced amino acid sequences from the cDNAs and their homologous comparison analyses were performed on PC/GENE 6.8.

1.5 Expression and purification of α-bungarotoxin (V31)

The synthetic oligonucleotide primers were designed to amplify the DNA fragments spanning the open reading frame of the mature variant, α-bungarotoxin (V31). The plus primer was 5′ − CCCGAATTCATCGTATGCCACACAACAG − 3′ and the minus primer was 5′ − CCCCTGCAGTTATCAACCAGGTCTCTGTTTC − 3′. *Eco*R Ⅰ site was introduced at 5′-terminus of the plus primer and *Pst* Ⅰ site at 3′-terminus of the minus primer. The amplified fragments were cut with both *Eco*R Ⅰ and *Pst* Ⅰ and then ligated into the large fragments of *Eco*R Ⅰ/*Pst* Ⅰ-cut pMAL-p2 vector. The resulting plasmid was transformed into *E. coli* BL21 strain. Transformats were selected on LB-agar plates with 100 μg/mL ampicillin. For induction of gene expression, the transformats were grown at 37℃ in LB medium containing 100 μg/mL ampicillin. After OD_{600} reached about 0.5, IPTG was added to a final concentration of 1 mmol/L and the culture was induced for a period of 4 h. The cells were harvested and lysed by supersonication. The expressed fusion protein was almost soluble in the supernatant. After centrifugation, the supernatant was applied to a sepharose 6B-amylose affinity column. The affinity resin was prepared according to the published method (Kroviarski, 1993). The purified fusion protein was digested by factor X_a (the ratio of X_a and fusion protein was 1∶100) in the buffer of 20 mmol/L Tris.Cl, 100 mmol/L NaCl, 2 mmol/L $CaCl_2$ (pH 8.0) at 25℃ for 16 h.

1.6 Immunoblotting of the purified fusion proteins

The balb/c mice were injected s.c. at multiple sites with low dose of natural α-bungarotoxin (Sigma, USA) in Freund's complete adjuvant and boosted at two week intervals with an increasing dose. The titer was checked by gel diffusion test. Animals were bled

9 days after the last injection. The dot blotting and the western blotting were performed in essentially the same manner as described in the protocol (Bio-rad, Inc., USA).

1.7 The *in vivo* toxicity assay

The Kun Ming mice weighing about 20 g were classified into five groups. Each group contained four mice. They were injected the recombinant α-bungarotoxin (V31) subcutaneously in different doses. Two mice were subcutaneously injected the maltose binding protein plus X_a as a negative control. The observation of death were undertaken during 24 h.

2 Results and Discussion

2.1 cDNA cloning of α-bungarotoxin (V31)

Comparative analysis on the determined cDNA sequences of α-neurotoxins, κ-neurotoxins and cytotoxins from Elapidae and Hydrophiidae venoms showed that the nucleotide sequences of the 5′, 3′-untranslated regions and the signal peptide coding region were highly conserved. Thus, the two degenerate primers were designed from the highly conserved regions. PCR amplification with the designed degenerate primers resulted in the isolation of the PCR fragments estimated to be approx. 300 bp by 2% agarose electrophoresis (data not shown). The DNA fragments were then purified by low melting point agarose and cloned into the pGEMT-vector. The white clones were screened by PCR and *Apa* I /*Pst* I restriction digestion. Then the positive clones were selected for nucleotide sequencing. By analysis of the sequenced cDNAs, the cDNA encoding a variant of α-bungarotoxin was cloned, in addition to the cDNAs of the neurotoxin-like proteins (Qian *et al.*,

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                                                                  60
AGATGAAAACTCTGCTGCTGTCCTTGGTGGTGGTGACAATCGTGTGTCTGGACTTAGGAT
  -21
   M  K  T  L  L  L  S  L  V  V  V  T  I  V  C  L  D  L  G
                                                                 120
ATACCATCGTATGCCACACAACAGCTACTTCGCCTATTAGCGCTGTGACTTGTCCACCTG
  -1 +1
Y  T  I  V  C  H  T  T  A  T  S  P  I  S  A  V  T  C  P  P
                                                                 180
GGGAGAACCTATGCTATAGAAAGATGTGGTGTGATGTATTCTGTTCCAGCAGAGGAAAGG

G  E  N  L  C  Y  R  K  M  W  C  D  V  F  C  S  S  R  G  K
                                                                 240
TAGTCGAATTGGGGTGTGCTGCTACTTGCCCTTCAAAGAAGCCCTATGAGGAAGTTACCT

V  V  E  L  G  C  A  A  T  C  P  S  K  K  P  Y  E  E  V  T
                                                                 300
GTTGCTCAACAGACAAGTGCAACCCACATCCGAAACAGAGACCTGGTTGAGTTTTGCTCT
                                                        +74
C  C  S  T  D  K  C  N  P  H  P  K  Q  R  P  G
                  319
CCTCCATCATGGACCATCC
```

Fig. 1 The nucleotide and deduced amino acid sequences of the precursor of the variant of α-bungarotoxin, α-bungarotoxin (V31)

The nucleotide sequence is shown above the deduced amino acid sequence including a 21 residue signal peptide indicated from −21 to −1 and a 74 residue mature protein starting from +1 to +74. The right end marks the positions of the nucleotides. The plus and minus primers are underlined.

1998). The cDNA encoding the mature α-bungarotoxin has recently been cloned from Taiwan banded krait (*Bungarus multicinctus*) by two steps of RT-PCR because of the complex secondary structure of the RNA (Kuo *et al*., 1995). However, the cDNA reported here was cloned by one step of RT-PCR and it has complete open reading frame with the initiation codon ATG and the termination codon TGA. The cDNA was 95.9% similar to that of α-bungarotoxin from Taiwan banded krait. The deduced precursor contains a 21 amino acid residue signal peptide and a following 74 amino acid residue mature protein. The signal peptide rich in hydrophobic amino acid residues resembles to those of short chain neurotoxins, κ-neurotoxins and cardiotoxins. The amino acid sequence of the mature protein is identical to α-bungarotoxin (V31), a minor variant of α-bungarotoxin identified by protein sequencing technique (Kosen *et al*., 1988).

Remarkably, two clones contained the cDNAs encoding the deletion precursor of α-bungarotoxin (Fig.2). The deleted fragment is 105 bp long. The deletion does not change the reading frame and the deduced deletion precursor has 60 amino acid residues with the deletion from position −2 to +33 of the amino acid sequence. Several genes encoding short chain neurotoxins and cardiotoxins have been identified. They are the ones for erabutoxin c from *Laticauda semifasciata* (Fuse *et al*., 1990); cobrotoxin, cobrotoxin b, cardiotoxin 4 and cardiotoxin 7 from *Naja naja atra* (Chang *et al*., 1997a, b and Y18014); α-neurotoxin ntx, cardiotoxin 3 from *Naja sputatrix* (AF096999 and AF064096). They have the similar gene organization which includes three exons separated by two introns. The exon 1 of all the genes ends at the positon (TTA G) corresponding to the signal peptide between amino acid residues −4 (Leu) and −3 (Gly). The length of exon 2 is 102 bp in the cardiotoxins; 105 bp in cobrotoxin b; 108 bp in erabutoxin c, cobrotoxin and the α-neurotoxin ntx. The deletion position of the cDNA reported here is exactly after the site (TTA G) and the length of the deleted fragment is about that of the exon 2 of the genes. It is suggested that the cDNA of the deletion precursor might be result from the direct linkage of exon 1 and exon 3 during RNA splicing. The same phenomenon was also found in the cDNA of cardiotoxin V from *Naja naja atra*, in which 105 bp was deleted at the same position (data not shown). The identification of the genes of α-bungarotoxin and the cardiotoxin V will help to explain the deletion phenomenon.

2.2 Expression and purification of α-bungarotoxin (V31)

The mutant, α-bungarotoxin (V31), was expressed as a fusion protein in *E.coli*. pMAL − p2 was adopted as the expression vector. The expression system has the advantage that it avoids the formation of insoluble inclusion bodies and the maltose binding protein is overexpressed. The plus and minus primers were designed to amplify fragments encoding the mature variant, α-bungarotoxin (V31). The amplified fragments were then cut with *Eco*R I and *Pst* I and ligated into the purified large fragments of *Eco*R I / *Pst* I -cut pMAL − p2. The constructed expression vector was transformed into *E.coli* strain BL21. The expressed fusion protein contained maltose binding protein and the following objective protein. There is a factor X_a cleavage site (IleGluGlyArg) between them. The objective product can be achieved after digestion by factor X_a. Since the maltose binding protein is overexpressed and soluble, the fusion protein was also overexpressed and mostly soluble in *E.coli* BL21. After supersonication, greater than 90% of the recombinant fusion protein was recovered in the supernatant following centrifugation of the extracts as judged by SDS-PAGE. The fusion protein was then purified by sepharose 6B-amylose affinity chromatography. The affinity resin could be easily prepared and the fusion protein was efficiently purified (Fig.3). The correctly expressed and purified fusion protein was confirmed by dot blotting and western blotting with the antisera against natural α-bungarotoxin (Fig.3). The purified fusion protein was first dialysized against factor X_a digestion buffer and then digested by factor X_a. Unfortunately, the

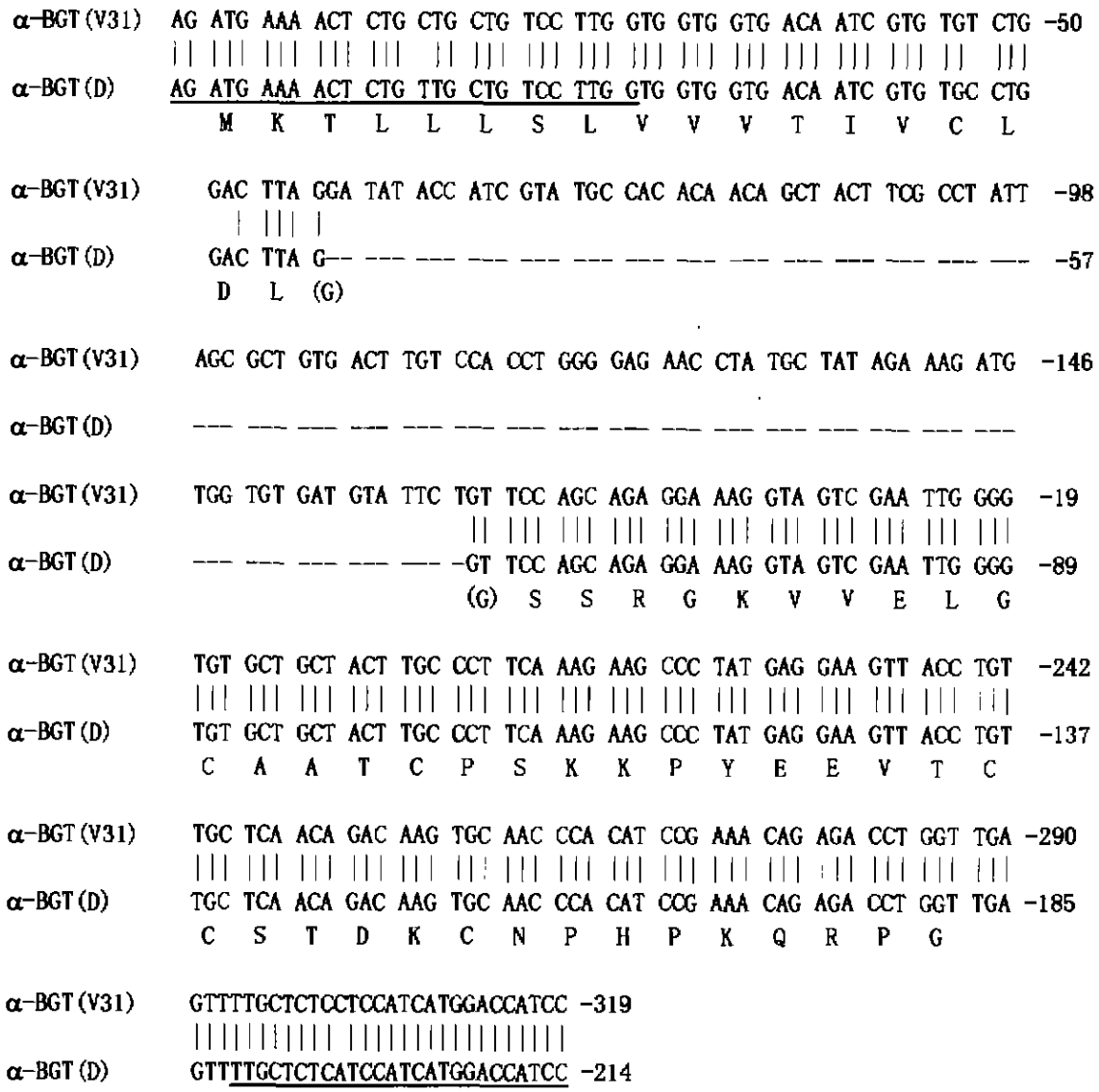

Fig.2 Comparison of the cDNA of the deletion precursor with that of the normal precursor of α-bungarotoxin (V31)
The cDNA of the deletion precursor of α-bungarotoxin is under that of the normal precursor of α-bungarotoxin (V31). The deleted fragment (105 bp) is marked with hyphens. The deduced amino acid sequence of the deletion precursor is also indicated in the single letter The plus and minus primers are underlined.

fusion protein was not digested. This phenomenon was also encountered by other researchers (Fiordalisi *et al*., 1991; Legros *et al*., 1997). It was thought that the factor X_a recognition site might be occluded. Thus the fusion protein was first dialysized against 8 *mol/L urea in absence of the reductant in case the* disulfide bonds were broken and then dialysized against factor X_a digestion buffer. After urea was removed by dialysis, the fusion protein could be mostly digested (Fig.3). The recombinant variant migrated in about the same position as that of the natural α-bungarotoxin in 15% SDS-PAGE.

2.3 *In vivo* toxicity of α-bungarotoxin (V31)

In order to understand whether the recombinant α-bungarotoxin (V31) is correctly folded, the *in vivo* toxicity assay was undertaken. The digested products of the purified fusion protein by factor X_a were taken directly to inject mice subcutaneously. At the same time, the purified maltose binding protein plus X_a was used as a negative control. The preliminary

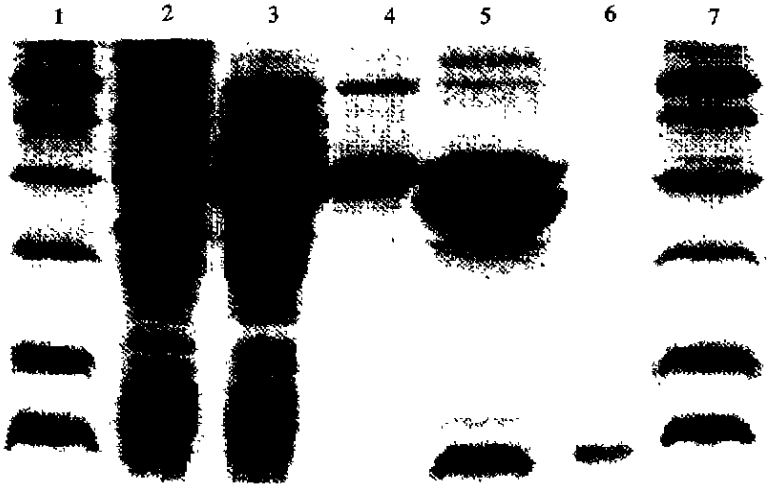

Fig.3 SDS-PAGE of α- bungarotoxin (V31)

Lane 2, the ununduced control; lane 3 and lane 4, the expressed and purified fusion proteins; lane 5, the digested purified fusion by factor X_a; lane 6, the natural α-bungarotoxin (purchased from Sigma, co.); lane 1 and lane 7, protein molecular weight markers in kD: 97.4, 66.2, 43, 31, 20.1 and 14.4.

Fig.4 Western blotting of α-bungarotoxin (V31)

Lane 1, the negative control of the purified maltose binding protein; lane 2, the purified fusion protein of α-bungarotoxin (V31) by sepharoe 6B-amylose affinity chromatography. The antisera was prepared against the natural α-bungarotoxin.

results showed that the toxicity of the recombinant variant was about 1/6 of that of the natural α-bungarotoxin (Table 1).

Table 1 The *in vivo* toxicity of the recombinant α-bungarotoxin (V31)

Group classification	1	2	3	4	5	Control
Fusion protein/μg	36	54	108	216	432	800
Recombinant protein/μg	6	9	18	36	72	0
Number of death	0	0	0	2	4	0
Rate of death/%	0	0	0	50	100	0

The toxicity of the natural α-bungarotoxin (Sigma, USA) in this test was in conformity with that reported (Mebs *et al.*, 1972) (LD_{50} = 0.3 μg/g, that is 6 μg per mouse).

The reason for the decrease of the toxicity could be explained as that there are four more amino acid residues in the N-terminus of the recombinant variant due to the limitation of the available expression vector. It was found previously that the 10 additional residues linked to the N-terminus of α-bungarotoxin decreased its affinity approx. 1.7-fold in the binding to AChR (Rosenthal *et al.*, 1994). However, the N-terminal three more residues of the recombinant kaliotoxin 2, a short polypeptide blocker of K^+ channels from scorpion venom, had 1 000-fold less affinity than the natural kaliotoxin 2 (Legros *et al.*, 1997). Anyway, the maltose binding protein expression system has the advantages that its fusion protein is overexpressed and mostly soluble in *E. coli* and the fusion protein can be easily purified. The yield of the purified fusion protein is about 65 mg/L culture. The functional overexpression of the recombinant variant suggest that the expression system will be useful for future site-directed mutagenesis to study the structure-function relationships of long neurotoxins.

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银环蛇神经毒素的分子克隆和功能表达

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摘要：抽提银环蛇毒腺总 RNA，通过反转录 PCR 技术扩增出其神经毒素 cDNA，克隆并测定了全序列。银环蛇神经毒素的 cDNA 编码 21 个氨基酸的信号肽和 74 个氨基酸的成熟蛋白。该信号肽非常类似于短链神经毒素、κ－神经毒素和心脏毒素的信号肽。而成熟蛋白的氨基酸序列与从台湾产银环蛇中通过蛋白测序鉴定的 α－bungarotoxin 的氨基酸序列完全一样。此外，还克隆到该神经毒素缺失前体 cDNA。利用麦芽糖结合蛋白融合表达系统，该神经毒素在大肠杆菌中得到高效的可溶性表达。表达的融合蛋白通过亲和层析得到有效纯化，并且通过蛋白印迹验证表达和纯化的融合蛋白。经过 X_a 因子酶切融合蛋白得到的重组神经毒素具有小鼠体内毒性，约为天然银环蛇神经毒素毒性的 1/6。该神经毒素的克隆和有效的功能表达为今后通过定点突变来研究长链神经毒素的结构与功能关系奠定了基础。

关键词：银环蛇神经毒素；cDNA 克隆；融合表达；小鼠体内毒性
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